MAIL STOP APPEAL BRIEF-PATENTS
PATENT
2005-1001

IN THE U. S. PATENT AND TRADEMARK OFFICE

In re application of

Willem Johan VAN DER GIESSEN et al. Conf. 9285

Application No. 10/089,460 Group 1615

Filed April 1, 2002 Examiner Carlos AZPURU

INTRALUMINAL DEVICE, COATING FOR SUCH DEVICE, AND METHOD FOR PREPARING SAID DEVICE

APPEAL BRIEF

MAIL STOP APPEAL BRIEF-PATENTS
Assistant Commissioner for Patents April 29, 2010
P.O. Box 1450
Alexandria, VA 22313-1450

MAY IT PLEASE YOUR HONORS:

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1. Real party in interest

The real party in interest in this appeal is:

ORBUSNEICH MEDICAL, INC., 1209 ORANGE STREET, WILMINGTON, DELAWARE 19801.

2. Related appeals and interferences

None.

3. Status of claims

Claim 3 has been cancelled. Claims 1, 2 and 4-18 are pending in this application and have been finally rejected, from which this appeal is taken.

4. Status of amendments

The Amendment filed subsequent to the final rejection has not been entered per the Advisory Action issued on April 7, 2010. The claims at issue are thus the ones set forth in the Amendment filed February 18, 2009.

5. Summary of claimed subject matter

Independent claim 1: As is set forth in independent claim 1, the present invention pertains to an intraluminal device, suitable for implantation in a body, which device is provided with a synthetic coating (Page 1, lines 3-4 and Page 6, line 20). The synthetic coating includes:

50-97% heparan sulfate; (Page 2, line 30)

1-20% laminin; and (Page 2, line 31)

0.2-15% type IV collagen. (Page 3, line 1)

Independent claim 6: As is set forth in independent claim 6, the present invention pertains to an intraluminal device, suitable for implantation in a body (Page 1, lines 3-4 and Page 6, line 20). The device is provided with a synthetic coating that includes:

50-97% heparan sulfate; (Page 2, line 30),

1-20% laminin; (Page 2, line 31),

0.2-15% type IV collagen; and (Page 3, line 1), and

an antibiotic (Page 4, lines 17-18).

Independent claim 7: As is set forth in independent claim 7, the present invention pertains to an intraluminal device, suitable for implantation in a body (Page 1, lines 3-4

and Page 6, line 20). The device is provided with a synthetic coating that includes:

50-97% heparan sulfate; (Page 2, line 30),

1-20% laminin; (Page 2, line 31),

0.2-15% type IV collagen; and (Page 3, line 1), and

an antibiotic including gentamycine (Page 4, lines 17-21).

Independent claim 12: As is set forth in independent claim 12, the present invention pertains to method for preparing an intraluminal device (Page 5, lines 27-28). The method includes the steps of:

- providing an intraluminal device for implantation in a body (Page 5, lines 29-30); and

- preparing a synthetic composition, including, in about 50 mg/ml solvent (Page 5, line 31):

50-97% heparan sulfate (Page 6, line 1),

1-20% laminin (Page 6, line 2),

0.2-15% type IV collagen (Page 6, line 3), where

the solvent is a suitable buffer or water (Page 6, line 4).

The method also includes:

- dipping the intraluminal device in the composition (Page 6, line 5); and

- drying the dipped intraluminal device (Page 6, line 6).

6. Grounds of rejection to be reviewed on appeal

The first ground of rejection for review on appeal is whether claims 1, 2, 4-6 and 8-18 would have been obvious over Schneider et al. (Journal of Vascular Surgery) within the meaning of 35 U.S.C. §103(a).

The second ground of rejection for review on appeal is whether claims 1, 2 and 4-18 fail to comply with the written description requirement within the meaning of 35 U.S.C. §112, first paragraph.

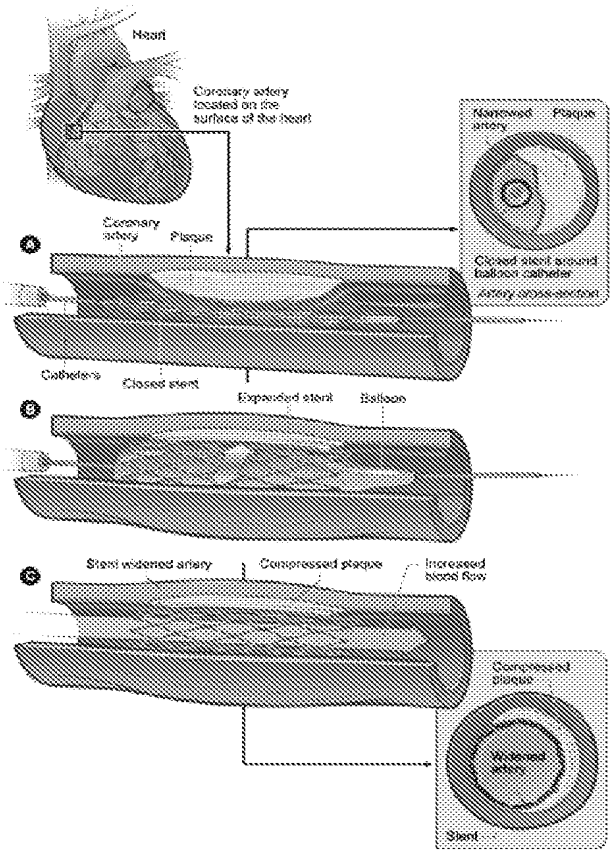
7. Argument

7.0 Summary of Argument

SCHNEIDER et al. does not teach or suggest a synthetic coating including 50-97% heparan sulfate. SCHNEIDER et al. teaches away from the synthetic coating of the present invention. The present invention demonstrates results that are unexpected over SCHNEIDER et al. Also, one of skill would recognize that the coating is synthetic from a proper understanding of the disclosure.

7.1 First Ground: Rejection Over Schneider et al.

The present invention is concerned with an intraluminal device (e.g. a stent) provided with a particular coating comprising several constituents in specific amounts. The coating performs under the rigorous conditions of stent utilization such as is illustrated below.



See:

http://www.nhlbi.nih.gov/health/dci/images/stent_lowres.gif

This particular coating improves vascular healing while also preventing thrombosis. Thrombosis is mainly prevented because of the use of heparan sulfate as a major constituent in the coating.

The amount of heparan sulfate used in the coating exceeds the amount found in the natural extracellular matrix (ECM). It was demonstrated this in the reply to the previous Official Action by referring to the document by NEVO et al., which was presented as evidence (see appendix).

This document of NEVO et al. demonstrates that the ECM produced by bovine corneal endothelial cells comprises a mere 3% heparan sulfate. Hence, the amount of heparan sulfate in the coating of the present invention (50-97%, as is set forth in the independent claims) is clearly non-natural.

In contrast, the document by Schneider et al., which is the only prior art document relied upon by the examiner for refusing the application, is concerned with a natural coating formed entirely of the above-discussed ECM produced by bovine corneal endothelial cells. There is no disclosure in SCHNEIDER et al. to use heparan sulfate as a major component in the coating in order to prevent thrombosis.

In fact, the coating of SCHNEIDER et al. includes a mere approximately 3% heparan sulfate, as evidenced by NEVO et al. However, the inventors found that an amount of heparan sulfate in the coating should be at least approximately 50% for sufficiently preventing thrombosis.

More particularly, According to NEVO et al. the total amount of proteoglycans in the ECM is 5% to 6% by dry weight percentage (see for example the abstract, p.51 first sentence or p.55 second paragraph). Of this total amount of proteoglycans in the ECM only 50% is heparan sulfate (see p.55 third paragraph). Thus, the ECM produced by bovine corneal endothelial cells is a mere 3% heparan sulfate.

In contrast, the present invention is concerned with a synthetic coating having heparan sulfate as the main constituent in a range of 50-97%. It was found that this coating has an improved anti-thrombogenic effect over the conventional coatings for intraluminal devices.

Therefore, the amount of heparan sulfate in the coating of SCHNEIDER et al. is not adequate for sufficiently preventing thrombosis.

The skilled artisan would not find any teaching or suggestion in Schneider et al. to increase the amount of heparan sulfate in the coating to a non-natural range of 50-97%. SCHNEIDER et al. argue against the use of a non-natural coating, since according to SCHNEIDER et al., the naturally produced ECM has superior cell growth-promoting properties as compared with isolated constituents. Page 655, right column, lines 45-47.

Therefore, SCHNEIDER et al. teach away from using a coating with specifically selected substituents departing from nature. A prior art reference must be considered in its entirety, i.e., as a whole, including portions that would lead away from the claimed invention. *W.L. Gore & Associates, Inc. v. Garlock, Inc.*, 721 F.2d 1540, 220 USPQ 303 (Fed. Cir. 1983), cert. denied, 469 U.S. 851 (1984). A *prima facie* case of obviousness may also be rebutted by showing that the art, in any material respect, teaches away from the invention. In

re Geisler, 116 F.3d 1465, 1471, 43 USPQ2d 1362, 1366 (Fed. Cir. 1997).

Moreover, SCHNEIDER et al. is concerned with a coating that improves adhesion and growth of ECs on synthetic graft material. In practice, the bare coating of SCHNEIDER et al. appears to have a proliferative effect with an enhanced probability of thrombosis. Not surprisingly, SCHNEIDER et al. teaches to expose the coating to glutaraldehyde or to seed vascular ECs to the coating to create a nonthrombogenic surface to prevent thrombosis. There is no disclosure or suggestion in SCHNEIDER et al. to modify the ECM in an attempt to improve the anti-thrombotic properties.

Furthermore, it is impossible to vary the types of constituents and the amounts thereof in a natural coating. The non-natural coating of the present invention however is optimized by selecting the specific constituents with beneficial properties in the specific amounts. Besides heparan sulfate, the other constituents are specifically selected to improve binding properties of the coating to the intraluminal device and to improve vascular healing. This particular selection of constituents surprisingly resulted in a highly efficient coating with improved properties. The resulting coating is not at all known from or rendered obvious by the cited prior art.

The present invention thus demonstrates results that are unexpected over the applied art of SCHNEIDER et al. These unexpected results would fully rebut any unpatentability that could be alleged.

Finally, the present invention is being rejected for unpatentability over a single reference. If a reference needs to be modified to achieve the claimed invention "there must be a showing of a suggestion or motivation to modify the teachings of that reference to the claimed invention in order to support the obviousness conclusion." *Sibia Neurosciences Inc. v. Cadus Pharmaceutical Corp.*, 225 F.3d 1349, 55 USPQ2d 1927 (Fed. Cir. 2000). The mere fact that references can be combined or modified does not render the resultant combination obvious unless the prior art also suggests the desirability of the combination. *In re Mills*, 916 F.2d 680, 16 USPQ2d 1430 (Fed. Cir. 1990).

In this case, SCHNEIDER et al. clearly teaches the desirability of natural coating, but the present invention utilizes a non-natural coating.

Therefore, SCHNEIDER et al. do not render the present invention *prima facie* unpatentable, and the unexpected results rebut any unpatentability that could be alleged. SCHMEDIER et al also teaches away from the present invention.

This rejection should accordingly be withdrawn.

7.2 Second Ground: Written Description

The independent claims of the present invention set forth a "synthetic coating" that includes "50-97% heparan sulfate."

The final Official Action of August 31, 2009 asserts that the claims set forth a "synthetic" composition that is not disclosed in the original specification, taking the position that the preparation of a composition as recited at page 6, line 20 of the specification is not adequate support, since even natural compositions such as those used in bone growth, must first treat the natural composition to isolate the desired product.

In asserting lack of written description, the Examiner must have a reasonable basis to challenge the adequacy of the written description. The examiner has the initial burden of presenting by a preponderance of evidence why a person skilled in the art would not recognize in an applicant's disclosure a description of the invention defined by the claims. *In re Wertheim*, 541 F.2d at 263, 191 USPQ at 97 (CCPA 1976).

In this case there is merely the assertion that any processing of a natural material would render it "synthetic."

However, the claims of the present invention are concerned with a synthetic coating containing 50-97% heparan sulfate. A naturally produced coating, i.e., a naturally produced ECM, contains a relatively low amount of heparan

sulfate, as evidenced by NEVO et al. Neither of these documents discloses or suggests a naturally produced ECM containing heparan sulfate in the range of 50-97%.

Therefore a coating comprising 50-97% heparan sulfate, which concentration is far removed from naturally occurring concentrations of heparan sulfate, will be understood by the skilled artisan to be non-natural, i.e., synthetic.

Also note the dependent claims, which claim antibiotics for example, which clearly would not arise from a natural sample.

Therefore it is respectfully submitted that the claims do comply with the written description requirement.

This rejection should accordingly be withdrawn.

8. Conclusion

The Appellant has demonstrated that the Examiner has failed to successfully allege that the rejected claims are new matter or *prima facie* unpatentable. It is clear that the claimed intraluminal device with coating present a truly inventive technology. For the reasons advanced above, it is respectfully submitted that all the rejected claims in this application are allowable. Thus, favorable reconsideration and reversal of the rejections of the under 35 USC §§112/103, by the Honorable Board of Patent Appeals and Interferences, are respectfully solicited.

Please charge the requisite Appeal Brief fee in the amount of $540 in which the fees are being paid online simultaneously herewith by credit card.

Respectfully submitted,

YOUNG & THOMPSON

/Robert E. Goozner/

Robert E. Goozner, Reg.No.42,593
Attorney for Appellant
209 Madison Street, Suite 500
Alexandria, VA 22314
Telephone (703) 521-2297
Telefax (703) 685-0573
(703) 979-4709

REG/jr

9. Claims Appendix

1. An intraluminal device, suitable for implantation in a body, which device is provided with a synthetic coating, wherein the synthetic coating comprises:

50-97% heparan sulfate;

1-20% laminin; and

0.2-15% type IV collagen.

2. The intraluminal device according to claim 1, wherein the coating synthetic comprises:

75-95% heparan sulfate;

3-10% laminin; and

0.5-10% type IV collagen.

4. The intraluminal device according to claim 1, wherein the synthetic coating further comprises a growth factor.

5. The intraluminal device according to claim 4, wherein the growth factor is selected from the group consisting of bFGF, IGF, TGF-β and VEGF.

6. An intraluminal device, suitable for implantation in a body, the device being provided with a synthetic coating that comprises:

50-97% heparan sulfate;

1-20% laminin;

0.2-15% type IV collagen; and

an antibiotic.

7. An intraluminal device, suitable for implantation in a body, the device being provided with a synthetic coating that comprises:

50-97% heparan sulfate;

1-20% laminin;

0.2-15% type IV collagen; and

an antibiotic comprising gentamycine.

8. The intraluminal device according to claim 1, wherein the synthetic coating further comprises vitronectine.

9. The intraluminal device according to claim 1, wherein the synthetic coating comprises:

85-95% heparan sulfate;

5-6% laminin;

3-4% type IV collagen;

0.5-1.5% entactin and nidogen;

0.001-1% growth factors; and

0.001-1% antibiotic.

10. The intraluminal device according to claim 1, wherein the intraluminal device is a prosthesis that comprises a stent or a graft.

11. A coating suitable for the intraluminal device according to claim 1.

12. A method for preparing an intraluminal device, comprising the steps of:

- providing an intraluminal device for implantation in a body;

- preparing a synthetic composition, comprising, in about 50 mg/ml solvent:

50-97% heparan sulfate;

1-20% laminin;

0.2-15% type IV collagen; and

the solvent being a suitable buffer or water;

- dipping the intraluminal device in the composition; and

- drying the dipped intraluminal device.

13. The method according to claim 12, wherein the synthetic composition further comprises entactin and nidogen.

14. The method according to claim 12, wherein the synthetic composition further comprises a growth factor, selected from the group consisting of bFGF, IGF, TGF-β and VEGF.

15. The method according to claim 12, wherein the synthetic composition further comprises an antibiotic.

16. The method according to claim 12, wherein the synthetic composition further comprises vitronectin.

17. The method according to claim 12, wherein the synthetic composition comprises:

85-95% heparan sulfate;

5-6% laminin;

3-4% type IV collagen;

0.5-1.5% entactin and nidogen;

0.001-1% growth factors; and

0.001-1% antibiotic.

18. The intraluminal device according to claim 1, wherein the synthetic coating further comprises entactin and nidogen.

10. Evidence Appendix

NEVO et al., *Connective Tissue Research*, 1984, Vol. 13, pp. 45-47, *Extracellular Matrix (ECM) Proteoglycans Produced by Cultured Bovine Corneal Endothelial Cells*.

Connective Tissue Research, 1984, Vol. 13, pp. 45–57
0300-8207/84/1301-0045/$18.50/0

EXTRACELLULAR MATRIX (ECM) PROTEOGLYCANS PRODUCED BY CULTURED BOVINE CORNEAL ENDOTHELIAL CELLS

ZVI NEVO,† ROBERT GONZALEZ and DENIS GOSPODAROWICZ

Cancer Research Institute and the Department of Medicine, University of California, Medical Center, San Francisco, CA 94143

(Received November 14, 1983; in final form January 25, 1984)

The nature of the proteoglycan(s) (PG) found in the extracellular matrix (ECM) layer produced by cultured bovine corneal endothelial (BCE) cells is analyzed. The PG(s) account for approximately 5 to 6% of the dry weight of the ECM, regardless of the amount of extracellular soluble PG available in the medium. A 4 M guanidinium chloride (GuCl) extract of ECM was separated on a dissociative cesium chloride (CsCl) gradient (1.25 g/cm^3 starting density). Results showed one main peak of PG substance(s) comprising 91% of the total labelled substance and uronic acid, banding at a specific buoyant density of 1.29 g/cm^3. The molecular weight of this major PG(s) as estimated by gel filtration on Sepharose CL-4B ranged from 0.5 to 0.7 × 10^6. Further chemical analysis of the main PG(s) band revealed a protein moiety accounting for 45% of the weight and carbohydrates-glycosaminoglycans (GAG) accounting for the remaining 55%. Analysis of the GAG chains (over the entire gradient) showed a composition, based on the susceptibility of the PG substance(s) to degrading enzymes, of 50% heparan sulfate, 43.5% dermatan sulfate, and 6.5% chondroitin 4- and 6-sulfate chains.

BCE cell cultures grown in the presence of β-*D*-xyloside produced an ECM lacking more than 90% of the GAG content found in the control ECM. The medium-soluble GAG chains, produced in vast excess in cultures grown in the presence of β-*D*-xyloside, are composed mainly of chondroitin 4- and 6-sulfates.

INTRODUCTION

For several decades it has been recognized that cells and their secreted matrices function as one dynamic unit whose components constantly interact with one another.[1–4] Bovine corneal endothelial (BCE) cells *in vivo* produce a thick extracellular matrix called Descemet's membrane.[5] *In vitro*, cultured bovine corneal endothelial cells retain their ability to produce large amounts of extracellular matrix (ECM) that uniformly coats the plastic substratum of culture dishes.[6–9]

Although the permissive effect of ECM on cell proliferation has been extensively studied,[6,9–14] the nature of the component(s) involved in such an effect is not known. Collagen, glycoproteins, and proteoglycans (PG) have been identified as the three major constituents of ECM isolated from organs as different in origin as kidney, lens and cornea[15–21] and of ECM produced by cultured cells.[14,22–26] A major advance in basement membrane chemistry was made when it was found that such a structure is produced by a murine tumor as an extracellular matrix, and is composed mostly of type IV collagen,[27] laminin[28] and heparan sulfate PG.[16] In the case of ECM produced by cultured BCE cells, studies have shown collagen types I, III, IV and V to be present in a ratio of 3:16:1.[26] The

†To whom reprint requests should be addressed at his permanent address: Dept. of Chemical Pathology, Tel Aviv University, Medical School Tel Hashomer 52621, ISRAEL.

major glycoproteins have been identified as fibronectin and laminin.[22] The nature of the major glycosaminoglycan (GAG) constituent is not presently known.

It is believed that the process of ECM formation involves a series of sequentially specific interactions among elements secreted by the cells (*e.g.* collagen and PG).[29–33] However, the precise order of events, the interacting components, and the variety of trapped substances still remain unknown. Changes in the amount of ECM produced, as well as alterations in its various components, could be a function of growth conditions, such as the presence or absence of growth factors, the quality and nature of the serum used to supplement the medium, the initial density at which the cells are seeded, and the passage number of cells (aging *in vitro*).[13] In the present study, we have partially characterized the PG present in ECM produced by cultured BCE cells.

MATERIALS AND METHODS

Dulbecco's modified Eagle's medium (DMEM, H-16) and glutamine were obtained from Gibco, Grant Island, NY. Fibroblast growth factor (FGF) was purified as described from bovine brains.[34] Calf serum and fetal calf serum were obtained from GIBCO, Grand Island, NY. Tissue culture dishes were from Falcon Plastics, Oxnard, CA. Hyaluronidase type IV, papain, Dextran T-40 (MW 40,000), chondroitin sulfate, hyaluronic acid, cetylpyridinium chloride (CPC), amino caproic acid, benzamidine hydrochloride, cesium chloride (CsCl), guanidinium chloride (GuCl) and *p*-nitrophenyl-β-*D*-xylopyranoside (β-*D*-xyloside) were obtained from Sigma Chemical Co., St. Louis, MO. Garamycin was purchased from Schering Corp., Bloomfield, NJ. Fungizone was from Squibb, New Brunswick, NJ. Blue dextrans (MW 5×10^5 and 2×10^6), Sephadex G-100, and Sepharose CL-4B were from Pharmacia, Piscataway, NJ. Chondroitinase ABC, chondroitinase AC-II, and keratanase were purchased from Miles Laboratories, Elkhart, IN. $Na_2^{35}SO_4$ (918 mCi/mM) and ^{3}H-glucosamine (D-1, 6-^{3}H-Gm, 45 Ci/mM) were obtained from New England Nuclear, Boston, MA. The water–bath sonicator was purchased from Laboratory Supplies Co., Hicksville, NY. Crude heparinase was the generous gift of Dr. Alfred Linker of the Veterans Administration Medical Center, Salt Lake City, UT. GAG standard markers were graciously provided by Dr. M. B. Mathews, University of Chicago, IL.

Cell Culture Conditions

Cultures of BCE cells were established from steer eyes as previously described.[35,36] Stock cultures were maintained on tissue culture dishes in DMEM, H-16 supplemented with 10% fetal calf serum, 5% calf serum, Garamycin (50 μg/ml), Fungizone (2.5 μg/ml), and 2 mM glutamine.

Preparation of ECM-Coated Plates

BCE cells were plated at an initial density of 10^5 cells/35 mm dish and maintained in the above medium supplemented with 5% (wt/vol) Dextran (40,000 MW). In some of the cultures β-*D*-xyloside was added to a final concentration of 2.5 mM.[37] (Unpublished preliminary studies indicated that Dextran T-40 does not affect PG synthesis or PG

distribution in BCE cells; a dose-response study of 0.025 to 5 mM β-D-xyloside showed that a concentration of 2.5 mM caused maximum inhibition of PG synthesis without seriously affecting cell growth). FGF (100 ng/ml) was added every other day until cultures became confluent (four to six days). The media were then renewed and the cultures further incubated for six days. The cultures were washed with phosphate-buffered saline (PBS), exposed for five min to 20 mM NH_4OH in distilled water, and rewashed with PBS five times, so that no cytoskeleton or nuclei could be observed associated with the intact ECM which coated the entire dish.

Determination of PG-GAG Production and its Distribution in Routine BCE Cell Cultures

One set of triplicate cultures was grown in the presence of 10 μCi $Na_2^{35}SO_4$ and subdivided at termination (as described below) into three fractions: medium, cells, ECM. The GAG-chain isolation procedure involved an exhaustive proteolysis step with papain, followed by dialysis against distilled water and CPC precipitation, as described in previous reports.[38–40]

A parallel set of triplicate cultures was used for examining cell morphology and cell counts. The morphological appearance of the cultures was analyzed by phase-contrast microscopy, and trypsinized cultures were counted with a Coulter Counter.

Isolation of ECM and its Chemical Analysis with respect to PG-GAG Molecules

ECM, PG, and GAG were isolated from BCE cultures seeded at a density of 6×10^5 cells/10 cm dish and grown under optimal conditions as described above. In addition to regular (unlabelled) cultures, some dishes were labelled with either $Na_2^{35}SO_4$ (75 μCi/plate) or ^{3}H-glucosamine (25 μCi/plate). Confluence was reached by the fourth day, and the cultures were maintained for an additional 12 days in the original medium before termination. At termination the cultures were collected as three separate fractions: (1) The medium was removed and combined with one wash of PBS (10 ml); (2) The cells were lysed with a hypotonic solution of 20 mM NH_4OH in distilled water (5 ml) and then combined with five washes of PBS (5 ml each); (3) The ECM was collected by three different procedures: For determination of total uronic acid concentration, ECM was removed from the plates by mechanical scraping. The material obtained was dialyzed against distilled water and then sonicated into a fine suspension in a waterbath sonicator. For determination of the chemical composition and molecular weight of the PG(s) an extract of 4 M GuCl containing antiproteolytic agents was obtained, as described by Oegema *et al.*[40] The extract was first dialyzed against 4 M GuCl. To yield monomeric PG molecules, a dissociative CsCl equilibrium gradient was established using Beckman Ultra-centrifuge Model L2-65B (Sw-41 rotor, 13 ml tubes) at 280,000 × g for 48 h at 20°C. Original attempts with a CsCl gradient of 1.50 g/cm^3 specific density (commonly used to separate cartilage PG) failed, because the PG component(s) banded at the top of the gradient without separating. When a gradient of lower buoyant density (1.25 g/cm^3) was used, the components entered and separated. Those PG(s) banded at the specific density of 1.29 g/cm^3 were then further analyzed.[16,40]

Molecular weight was determined by gel chromatography utilizing a Sepharose CL-4B column (82 × 2 cm) equilibrated and eluted with 4 M GuCl in 0.02 M Tris-HCl buffer (pH 7.0) at a rate of 10 ml/h blue dextrans and PG monomers from Swarm rat chondro-

sarcoma[39,40] and mouse sarcoma[16,41] (prepared by GuCl extraction and CsCl dissociative equilibrium gradients starting with 1.50 and 1.35 g/cm^3) were used as molecular weight markers. Relative protein, uronic acid, and hexosamine concentrations were determined by methods described below. For analysis of the GAG chains, mechanical scraping of ECM was followed by papain digestion (2 mg/plate). The ECM-papain digest was then further processed, as described by Nevo,[38] to yield isolated GAG chains.

The GAG chains were further purified and characterized by enzymatic digestion overnight at 37°C, using the following enzymes:[42,43] crude heparinase preparation (0.2 mg crude heparinase in 0.1 M sodium acetate at 30°C); keratanase (keratanase in 0.05 M Tris-HCl buffer, pH 7.4); chondroitinase ABC (0.2 units chondroitinase ABC in 0.03 M Tris-acetate buffer, pH 8.0, containing 0.25 M NaCl); chondroitinase AC (0.2 units chondroitinase AC-II in 0.1 M Tris-HCl buffer, pH 7.4); and hyaluronidase (1 mg hyaluronidase type IV-S in 0.1 M monosodium phosphate buffer, pH 5.2, containing 0.15 M NaCl). GAG standard markers were used to ascertain the proper action of the chemical treatment and the enzymatic digests.

Nitrous acid treatment of GAG preparations was performed as follows: one volume of 5% $NaNO_2$ and one volume of 33% acetic acid were added to one volume of GAG sample and incubated for 90 min at room temperature (20°C). The reaction was terminated by adding one volume of 12.5% ammonium sulfamate.[44]

The enzymatic digests and the hydrolysates were further separated by gel chromatography on a Sephadex G-100 column (65 × 1.6 cm) equilibrated and eluted with PBS at a rate of 15–20 ml/h. The column was calibrated for the void volume region using dextran blue (MW 2 × 10^6), chondroitin sulfate, and hyaluronic acid, and for the GAG-oligomer region using the partially degraded products of chondroitin sulfate resulting from short exposure to chondroitinase ABC, or those of hyaluronic acid exposed to hyaluronidase. The final products of the above digests were used for locating the tetramer-dimer region. Chromatography was followed by colorimetric analysis to measure uronic acid concentration.

Chemical determinations were performed on the preparations of ECM, PG, GAG and their products resulting from either the enzymatic or the chemical treatment. These included: Lowry's protein assay as modified by Maxwell, *et al*,[45] uronic acid determination, as originally described by Dische and modified by Bitter and Muir;[46] determination of hexosamines[47] and unsaturated disaccharides.[48] For radioactivity measurements, Aquasol-2 cocktail of New England Nuclear was used as the scintillation fluid, and samples were counted in a Beckman LS8000 liquid scintillation counter.

RESULTS

Yields and Gross Analysis of ECM

BCE cells seeded on a 10 cm dish yielded 3.9 mg of powdered ECM per plate, or approximately 50 μg/cm^2 or 650 pg/cell. Uronic acid was present in the ECM suspension at a concentration of 0.9% by weight.

Isolation and Characterization of PG(s) Obtained by CsCl Equilibrium Gradient

The parallel profiles of both ^{35}S- and ^{3}H-glucosamine labelled substance(s) resulting from the CsCl equilibrium gradient are illustrated in Figure 1. Both show only one major peak, around 1.29 g/cm^3, comprising 91% of the radiolabelled materials, and possibly a minor peak at the bottom of the gradient, comprising the remaining 9% of the radiolabelled materials. A similar profile with regard to density and quantity was observed following the determination of uronic acid distribution in the unlabelled extract of ECM. The PG peaks

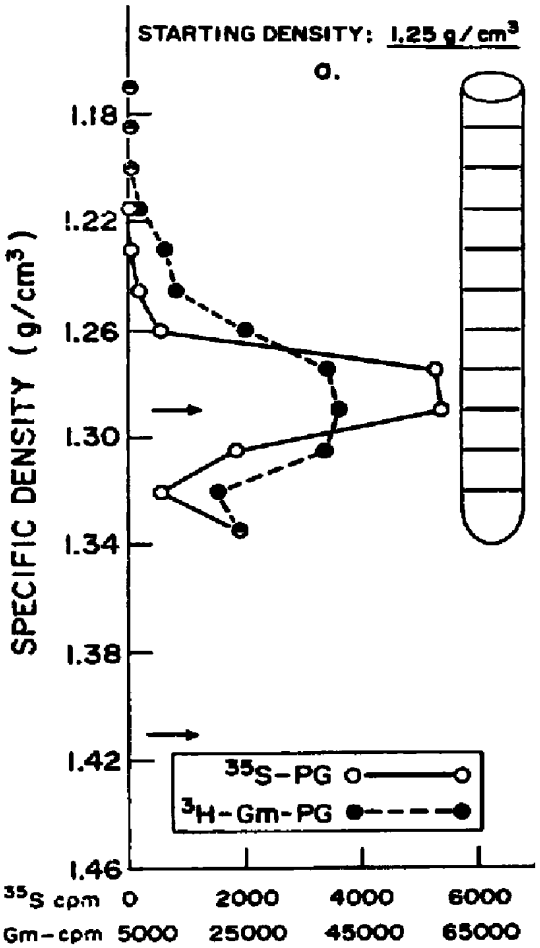


FIGURE 1 Banding of PG(s) from ECM of BCE cells on CsCl dissociative density gradient (starting density 1.25 g/cm^3). Radioactivity was followed in the gradient fractions of separated ECM extracts of cultures grown in the presence of radiolabelled precursors (Gm = Glucosamine). Uronic acid and specific density (weight/volume) were determined in the cold gradients.

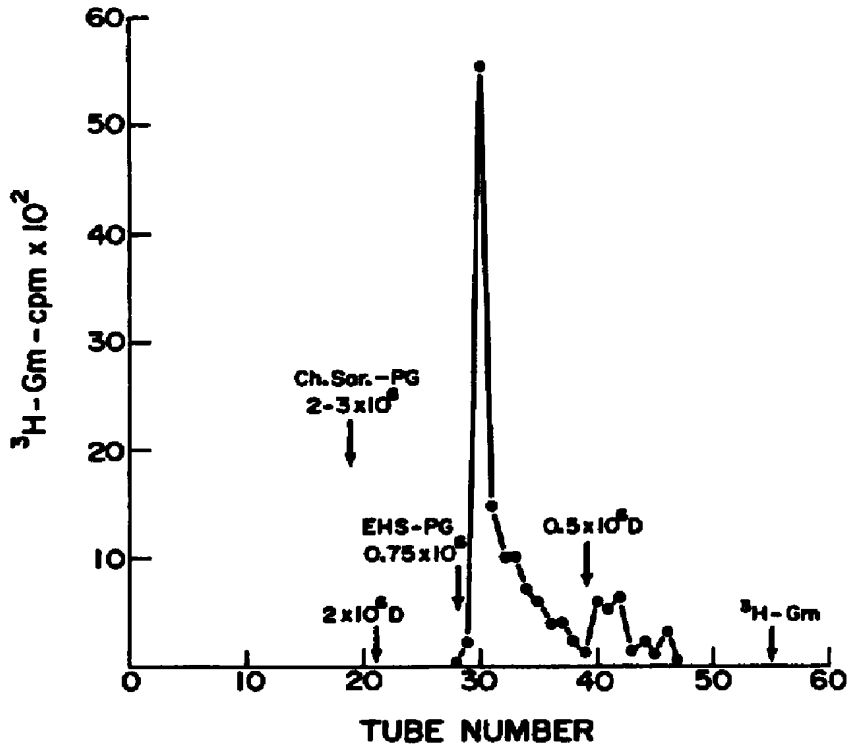


FIGURE 2 Elution profile of the major ^{3}H-glucosamine (Gm)-PG peak obtained on a CsCl gradient as chromatographed on Sepharose CL-4B, added in 1.0 ml of 4 M GuCl and eluted by a dissociative solution of 4 M GuCl containing buffer and antiproteolytic agents.

banding at the density of 1.29 g/cm^3 of the labelled and unlabelled material were collected separately, dialyzed, and used for further characterization. The PG(s) banding at the bottom of the gradient could not be further investigated due to the limited amounts.

To determine molecular weight, 1 ml samples were applied on the Sepharose CL-4B column and eluted with 4 M GuCl in buffer. Figure 2 shows the elution sites of ^{3}H-glucosamine-PG and those of the blue dextrans (MW 0.5 × 10^6 and 2 × 10^6) and the Swarm rat chondrosarcoma and Engelbreth-Holm-Swarm (EHS) mouse sarcoma PGs (MW 2–3 × 10^6 and 0.75 × 10^6, respectively). As calculated on the basis of the elution profiles of the PG markers, the results of ^{3}H-glucosamine-PG chromatography indicate the presence of one major peak eluting in the molecular weight range of 0.5–0.7 × 10^6 daltons. A similar profile was observed when ^{35}S-PG was analyzed under similar conditions (unpublished data).

Further chemical analysis of the unlabelled PG banded at the specific density of 1.29 g/cm^3 (originating from 100 mg of powdered ECM) revealed that it was composed of 45% protein, 15% uronic acid and 15% hexosamines by weight. The remaining 25% probably consisted of sulfate, suggesting an average of 1.7 moles sulfate/disaccharide unit. From the ratio of uronic acid content in ECM (0.9% by weight) and the above figures for PG, it can be roughly estimated that sulfated GAG accounts for 3% of the dry weight

of ECM and PG for 5–6%. The calculated figures presented in this paragraph may slightly change if the PG fraction banded at the specific density of 1.29 g/cm^3 is contaminated with other minor non-PG macromolecular components, also double labelled by ^{35}S and ^{3}H-glucosamine, such as sulfated glycoproteins, other than keratansulfate, proven to be absent by keratanase.

Characterization of GAG Chains

GAG chains were isolated from a papain digest of ECM which included the PG bands at the specific densities of 1.29 g/cm^3 (91%), and the bottom band (9%).

Gel chromatography on a Sephadex G-100 column was used to separate the various isolated GAG chains from their degradation products after enzymatic or chemical treatment (Figure 3). Figure 3A shows the control profile of an untreated ^{35}S-GAG chain, with one major peak eluted near the void volume. This indicates that the GAG chain population(s) had a molecular weight higher than 1×10^4. The ^{3}H-glucosamine-GAG showed a parallel distribution. The entire papain digest of ^{35}S-PG from ECM, when applied on the column without prior dialysis, contained, in addition to a major peak eluting in the void volume, small amounts (less than 5% of the input) of heterogeneous labelled components with a molecular weight lower than 1×10^4. These low molecular weight components, which are eliminated during the isolation procedure of GAG chains, are probably the products of either partial enzymatic degradation or unidentified sulfated glycoproteins. Hence, more than 95% of the GAG in ECM is composed of high molecular weight chains, migrating at the void volume on Sephadex G-100 column.

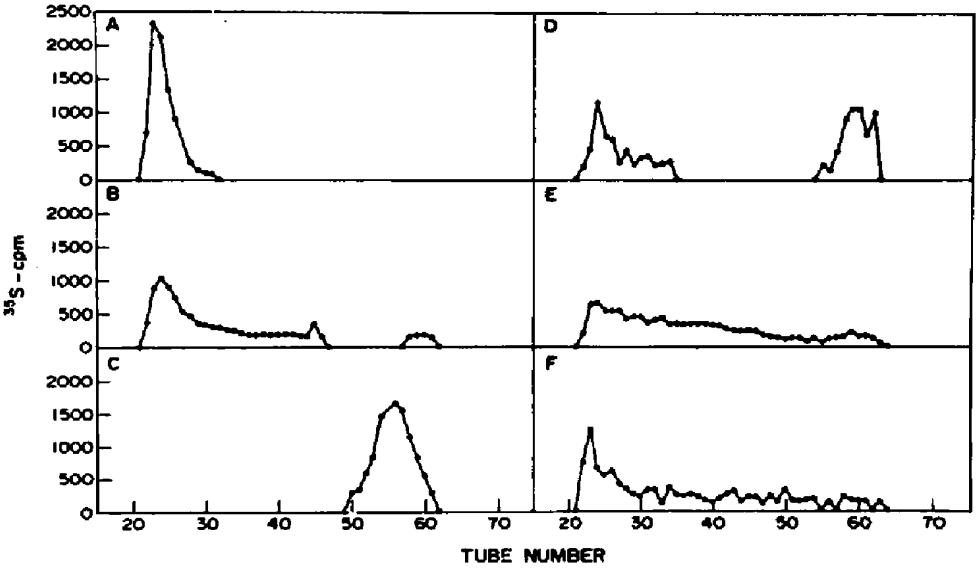


FIGURE 3 Chromatographic profiles on Sephadex G-100, of chemically treated, enzymatically treated, or untreated ^{35}S-GAG (12,000 total cpm) equilibrated and eluted by PBS. (A) control sample of untreated ^{35}S-GAG; (B) nitrous acid-treated ^{35}S-GAG; (C) crude heparinase-treated ^{35}S-GAG; (D) chondroitinase ABC-treated ^{35}S-GAG; (E) chondroitinase AC-treated ^{35}S-GAG; (F) testicular hyaluronidase-treated ^{35}S-GAG.

Nitrous acid treatment of the ^{35}S-GAG ECM caused a 50% degradation of the peak appearing in the void volume. This correlated with the appearance of highly heterogeneous oligomers of lower molecular weight (Figure 3B). Treatment of ^{35}S-GAG with a crude heparinase preparation (containing chondroitinase activities) resulted in the complete disappearance of the peak appearing in the control at the void volume and a subsequent shift of the degraded labelled substances to the eluting region of the dimers (Figure 3C). The elution profile of keratanase-treated ^{35}S-GAG was identical to that shown in Figure 3A. This suggests that no degradation due to keratanase occurred and that keratan sulfate chains were not present.

Chondroitinase ABC treatment of ^{35}S-GAG cleaved approximately 50% of the labelled chains from the void volume peak and led to their subsequent appearance at the dimer-eluting region (Figure 3D). Treatment with chondroitinase AC also resulted in the degradation of approximately 50% of the peak originally present in the void volume; however, in this case the degraded products were distributed heterogeneously along the profile and trailed towards the dimer-eluting region, thereby indicating the presence of dermatan sulfate (Figure 3E). Measurements carried out on the chromatographic profile of the chondroitinase AC digest of the ^{35}S-GAG-ECM fraction (Figure 3E) provided a general indication of GAG chain composition. The profile was divided into three regions: (a) void volume (50% of the radioactivity); (b) oligomer region (43.5%); and (c) dimer region (6.5%). These measurements, together with the evaluation of the chromatographic profiles of ^{35}S-GAG-ECM subjected to nitrous acid, heparinase, and chondroitinase ABC digestion, suggest that heparan sulfate-type molecules account for 50% of the GAG, dermatan sulfate for 43.5%, and chondroitin 4- and 6-sulfates for 6.5%.

Testicular hyaluronidase treatment of ^{35}S-GAG-ECM resulted in a chromatographic profile resembling that observed after treatment with chondroitinase AC (Figure 3F). Only traces of radioactivity were present in the region where tetramers and dimers were eluted. However, the elution profile of ^{35}S-GAG treated with hyaluronidase and that treated with chondroitinase ABC differed significantly. The former was composed of a massive fraction of partially degraded oligomers, whereas the chondroitinase ABC digest was composed of fully degraded GAG in the form of dimers. In both digests, 50% of the labelled GAG was eluted at the void volume region. This further confirms the observation that, in addition to heparan sulfate, another GAG rich in iduronic acid, probably dermatan sulfate (known to be hyaluronidase-resistant), is present. Colorimetry[48] performed on parallel enzyme digests of unlabelled GAG-ECM indicated the presence of unsaturated residues in the non-reducing ends of chondroitinase ABC and heparinase GAG digests, but only traces of unsaturated residues were detected in the chondroitinase AC digest.

Source and Nature of the GAG Integrated into the ECM Structure

Table I shows the incorporation and distribution of ^{35}S into GAG molecules present within the cells, secreted into the medium, or found in the ECM produced by cultures seeded at a high initial cell density (10^5 cells per 35 mm dish). It can be seen that in cultures grown on either plastic or ECM-coated dishes, there were only minor differences in the concentration of ^{35}S-GAG found in the various fractions. Marked differences emerged, however, when cultures were seeded on plastic and exposed to *β-D*-xyloside, which acts as a GAG chain acceptor or chain initiator, competing with the xylosylated core protein and blocking normal PG synthesis. In these cases 97% of the ^{35}S-GAG was found in the medium, 2.7%

TABLE I

Distribution of ^{35}S-GAG molecules among the three BCE cell fractions under three growth conditions

	Plastic	Complete ECM	Plastic + β-D-xyloside
Cell counts (× 10^5 ±SD) at termination	7.3 ±0.16	9.7 ±0.36	4.5 ±0.04
cpm in medium ^{35}S-GAG per 10^5 cells (% of total)	1495 ±89(30.4)	2495 ±728(41.1)	27019 ±1429(97.0)
cpm in cellular ^{35}S-GAG per 10^5 cells (% of total)	2371 ±354(48.2)	2807 ±344(46.3)	739 ±80(2.7)
cpm in ECM-^{35}S-GAG per 10^5 cells (% of total)	1049 ±30(21.4)	761 ±96(12.6)	89 ±50(0.3)

BCE cells seeded at a density of 100,000 cells per 35 mm dish and grown with FGF in the presence of 10 μCi $Na_2{}^{35}SO_4$ reached confluence on the fourth day and were maintained for seven days. Cultures were run in two sets of triplicates, one set for cell counts and one set for radiolabelling and isolation of ^{35}S-GAG chains. The figures represent the mean ± standard deviation. The sum of the figures in parentheses (% of total), for medium, cells, and ECM of each substratum is 100%.

in the intracellular pool and only 0.3% in the ECM. This suggests that ECM produced by BCE cells exposed to β-D-xyloside (X-ECM) is devoid of most of the GAG content normally found in ECM produced by cells which are not exposed to this compound.

Analysis of the ^{35}S-GAG released into the medium of cultures grown in the presence of β-D-xyloside (Figure 4) was conducted as previously described for ^{35}S-GAG from ECM

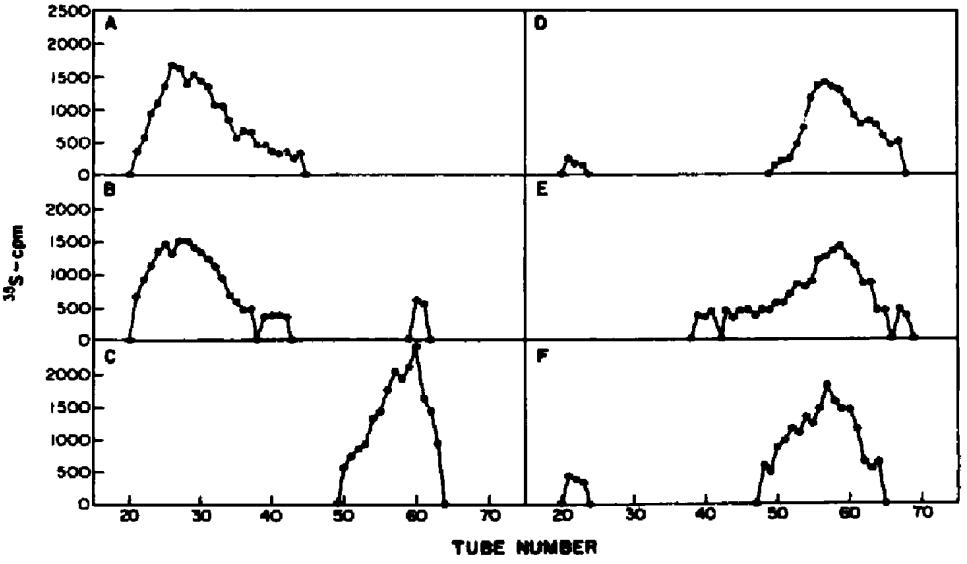


FIGURE 4 Sephadex G-100 column chromatography of glycosaminoglycans isolated from the medium of BCE cell cultures grown in the presence of β-D-xyloside and treated with nitrous acid or various enzymes. Isolated glycosaminoglycans (A), GAG treated with nitrous acid (B), crude heparinase (C), chondroitinase ABC (D), chondroitinase AC (E), or testicular hyaluronidase (F). Samples were then applied on a 65 × 1.6 cm column of Sephadex G-100 equilibrated in PBS. Fractions of 1.5 ml were collected and assayed for radioactivity.

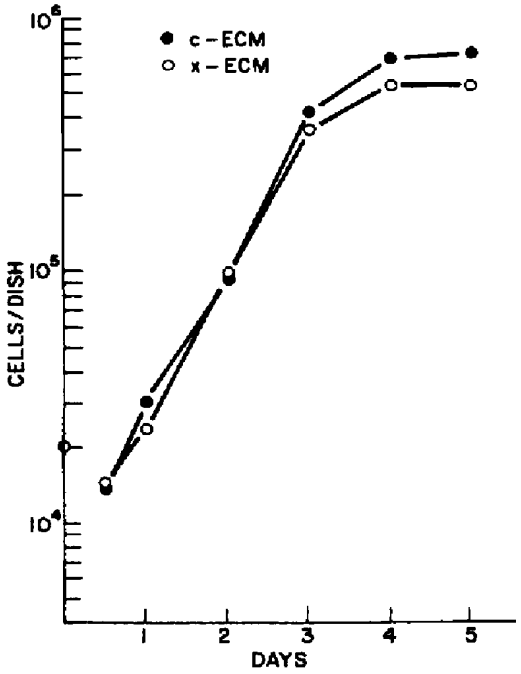


FIGURE 5 Growth curves of BCE cell cultures grown on complete (c) ECM and produced in the presence of β xyloside (x) ECM.

(Figure 3). The profile of untreated ^{35}S-GAG showed one major peak eluting at the void volume (Figure 4A). Nitrous acid treatment degraded a minute amount of the labelled material (Figure 4B) suggesting that virtually no heparan sulfate chains were present. Treatment with crude heparinase, chondroitinase ABC, chondroitinase AC, and hyaluronidase (Figure 4C-F) caused complete degradation of the void volume peak into low molecular weight substances (tetramers or dimers) (Figure 4C-F); this suggests the absence of dermatan sulfate and the presence of chondroitin sulfates. Hence, the PG-GAG products of BCE cells grown in the presence of β-*D*-xyloside are probably not readily integrated into the ECM, as are PG molecules of regular (control) cultures.

The growth curves described in Figure 5 were designed to compare the growth-rates of BCE cell cultures grown on complete ECM vs x-ECM. Cell growth was followed for five days; confluence was reached by the fourth day. The growth-rates appear to be identical for the first three days of exponential growth. Differences begin to emerge on the third day, when the rate of cell proliferation was slower on x-ECM. The differences increased by the fifth day, at termination the final cell density on x-ECM was 72% that of the control cultures grown on complete-ECM. These findings suggest that the GAG found in the ECM does not play a role during the initial exponential phases of growth, while their possible function becomes apparent in confluent cultures, since in their absence increases in cell size and lower final cell densities are observed.

DISCUSSION

A vast body of information is available describing the specific and nonspecific interactions that occur among the various components forming the three-dimensional network structure of biomatrices produced by various tissues.[1,3,29,31–33,40] The extracellular matrix produced *in vivo* by various epithelial cells, including endothelial cells, is known by the term "basement membrane."[18,19,21,50] When grown *in vitro,* these same cell types, depending on the culture conditions and the type of substrate upon which the cells rest, can form similar extracellular structures, variously termed "exoskeleton," "cells' carpet," "cells' fingerprints," "microexudates," and "ECM."[6,13,51,52] Collagen, glycoproteins, and PGs have been identified as the three major constituents of these *in vivo* and *in vitro* structures.[16,24,31,50,53] The present communication deals with the nature of the PG(s) synthesized by cultured BCE cells and present in the ECM.

The total amount of PG found in the ECM produced in this study (5–6% by weight) was higher than that reported for other types of basement membranes isolated from tissues, such as the lens capsule.[21] This is in agreement with previous findings that cells in culture produce excessive amounts of matrix.[4]

Separation of the 4 M GuCl extract from the ECM under dissociative conditions on a CsCl equilibrium gradient gave rise to one major PG band migrating to the specific density of 1.29 g/cm^3. Further purification of this band by column gel chromatography resulted in one PG peak eluting in the range of 0.5–0.7 $\times$ 10^6 daltons. However, these two purification steps are inadequate for a conclusive proof of the presence of only one major type of PG. Moreover, total GAG chain analysis indicated the presence of two major types of chains, heparan sulfate (50%) and dermatan sulfate (43.5%), in addition to chondroitin 4- and 6-sulfate chains (6.5%). The two major chains have not been previously reported to share a common core protein; on the contrary, in cultures of human skin fibroblasts they were bound to two distinct core proteins.[54] Nevertheless, the heparan and dermatan sulfates share a common buoyant density and possess a similar molecular weight. The chondroitin 4- and 6-sulfate chains may be derived from the minor PG peak, banded on the CsCl gradient (1.25 g/cm^3) at the bottom of the tube. Thus, the partial degradation of dermatan sulfate chains by hyaluronidase treatment leading to production of oligomers of decreased molecular weight, would suggest the presence of co-polymer chains of dermatan and chondroitin sulfates. This combination of heparan and dermatan sulfates in the GAG chain population may contribute to certain unique molecular properties.[49] The possibility of the presence of one, two or more PGs in ECM is presently under investigation in our laboratory. Therefore, this question is left open till conclusive results can be obtained.

Although the buoyant density and the high protein content in the ECM-PG might define it as a glycoprotein, other analytical features conclusively demonstrate that it is, in fact, a PG. These include the presence of sugar side-chains with a xylose residue at the initiating point (as shown by the ability of β-*D*-xyloside to compete with and block its synthesis) (Table I), as well as the content and ratio of uronic acid to hexosamine. In addition, the molecular weight, density, and protein content of these PG(s) resemble those of PG(s) isolated from EHS mouse sarcoma,[41] known for its rich production of basement membrane components. On the other hand, quite a different composition was attributed to the synthetic products of rabbit corneal endothelial cells.[55]

To judge from the low concentrations of PG integrated into the ECM regardless of the amount of soluble PG available in the medium (*e.g.* cells maintained on plastic vary widely from cells grown on ECM-coated dishes in their ability to synthesize and to release PG(s) into the medium), it seems that ECM production is a self-limiting process that reaches a steady state.[1,56] This process may be controlled by one of the interacting components which is present at the lowest concentration in the medium and which acts as the rate-limiting factor.[30]

The effect of xyloside on PG synthesis was studied in detail in cartilage cell systems.[57,58] Most authors have found that xyloside inhibits synthesis of chondroitin sulfate PG,[57–59] whereas in the present study, xyloside seemed to inhibit heparan sulfate and dermatan sulfate chains as well. Stimulation of synthesis of free chains (free of core protein) attached to xyloside is certain for chondroitin sulfate, but controversial for heparan sulfate.[60–62] BCE cultures grown in the presence of xyloside produce PG-GAG molecules (97% in the medium) (Table I) which fail to integrate into the ECM structure. Although analysis of these cultures revealed the presence of only chondroitin sulfate chains (Figure 4) (most probably free chains), further investigations are necessary to explain the molecular failure which prevents their integration into the ECM structure, i.e., whether it is due to the formation of improper GAG chains on the core protein, or to the absence of the protein core itself. Xyloside is believed to affect specifically PG synthesis, as only minor changes were detected in collagen and other matrix components in its presence.[63] BCE cells adhesion seems not to be affected by the PG-GAG molecules of the ECM, whereas cell size and final cell density appear to be influenced by these molecules.

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11. Related Proceedings Appendix

None.